Supplementary Information

Potent STING Activation Stimulates Immunogenic Cell Death to Enhance Antitumor Immunity in Neuroblastoma

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Figure S1: Integrated molecular analysis of mRNA expression of genes from the Pediatric Neuroblastoma TARGET dataset. Genes are distinguished by functional significance and clustered based on high (upper tertile, n=47), intermediate (median...
tertile, n=47), and low (bottom tertile, n=47) TMEM173 mRNA expression. P values represent statistical significance after performing Dunn’s multiple comparisons tests to compare mRNA expression of the indicated genes in TMEM173 low versus TMEM173 intermediate versus TMEM173 high tumors.

Figure S2: Interferon-stimulated genes (ISG) and STING pathway related mRNA expression in MYCN non-amplified and amplified samples profiled by microarray in the TARGET Pediatric Neuroblastoma (n=55) datasets. Data were accessed through the cBioPortal (41). Mann-Whitney U test (two-tailed) was used for statistical comparison.
Figure S3: STING expression in neuroblastoma. A. A comparison of TMEM173 mRNA expression z-scores among all cancer tumor types profiled by microarray data in the Cancer Cell Line Encyclopedia (n=942). The list of abbreviations shown are: DLBCL: diffuse large B cell lymphoma; B-ALL: B cell acute lymphoblastic leukemia; NSCLC: non-small cell lung cancer; T-ALL: T cell acute lymphoblastic leukemia; CML: chronic myelogenous leukemia; AML: acute myelogenous leukemia. B. Basal STING expression in neuroblastoma cell lines by western blot analysis.

Figure S4: qRT-PCR gene expression of IFNB1, CXCL10, and TNF in neuroblastoma cell lines at 6, 24 and 48 hrs after treatment with vehicle (PBS), free cGAMP, empty nanoparticles (NP) or STING-NPs.
**Figure S5: Quantification of STING-NP-mediated apoptosis.** Flow cytometry dot plots for annexin V and 7-AAD staining after treatment with Vehicle (PBS), empty NPs, cGAMP (200 nM) and STING-NPs (200 mM cGAMP) for 48 hr in Neuro-2a and 9464D cells. Bar chart represent the percentage (mean ± SD) of live cells (7-AAD− and Annexin V−); early apoptotic cells (7-AAD− and annexin V+); late apoptotic cells (7-AAD+ and Annexin V+); and necrotic cells (7-AAD + and Annexin V−), respectively. Triplicate samples for three independent experiments.
Figure S6: Effect of STING-NP treatment on 9464D tumor microenvironment. 9464D cells were injected subcutaneously and allowed to grow to 200 mm\(^3\). Tumors were treated with PBS or STING-NPs (10 µg) every 3 days (3 injections total) and harvested at 48 hr from last treatment. A. qRT-PCR analysis of Ifn\(\beta_1\), Tnf, Cxcl10, and Il12 gene expression in injected 9464D tumors. n= 5-7 mice per group represented as mean ± SEM, *p<0.05, **p<0.01 indicate statistically significant difference using a two-tailed Mann–Whitney U-test. B. 9464D tumor lysates (n=3) were analyzed using western blot for IRF3, phospho-IRF3, PD-L1, caspase 3 and cleaved caspase 3. Gel loading was normalized for equal actin. The density of bands is shown under each immunoblot after normalization to actin. C. Immunohistochemical staining of tumor sections for cleaved caspase-3 (apoptosis), and CD8\(^+\) T cells; corresponding quantification of staining intensity using ImageJ software. Data shown as mean ± s.d. for n=3 9464D tumors, ***p<0.005, ****p<0.001 indicate statistically significant difference using a student t-test. D. qRT-PCR analysis of CD274 (PD-L1) gene expression in injected 9464D tumors; HMBS (hydroxymethylbilane synthase) is the house keeping gene. n= 3 mice per group represented as mean ± SEM, **p<0.01 indicate statistically significant difference using a two-tailed Mann–Whitney U-test. E. Flow cytometric analysis of PD-L1 expression on 9464D cells treated 24 h with STING-NP, free cGAMP, or PBS (vehicle) (***p<0.005 indicate statistically significant than PBS and cGAMP via student t-test).
**Figure S7**: Change in body weight of mice bearing Neuro-2a or 9464D NB tumors in response to intratumoral injection of indicated formulation or PBS. Mice were intratumorally administered cGAMP or STING-NPs at a dose corresponding to 10 μg cGAMP on days 9, 12, and 15 (Neuro2a) or days 14, 17, and 20 (9464D).

**Figure S8**: Gating strategy for flow cytometric analysis of calreticulin on NB cells. Following discrimination of single cells, live (PI negative) cells were gated on and the MFI of the CRT^+ was determined and normalized to the PBS (vehicle) treated control.
Figure S9: Gating strategy for flow cytometric analysis of dendritic cell uptake of CMFDA-labeled NB cells. Following discrimination of single cells, live (SYTOX Blue negative) CD11c⁺ were gated on, and the percentage of the CD11c⁺ population that were positive for CMFDA was determined.
Figure S10: Gating strategy for flow cytometric analysis of CD80 and CD86 expression by dendritic cells. Following discrimination of single cells, live (SYTOX Blue negative) CD11c+ were gated on, and the percentage of the CD11c+ population that were MHC-II*CD86+ or MHC-II*CD80+ was determined.